II. REMARKS

Claims 118, 141 and 163 have been amended herein to correct typographical errors and to clarify the claims. Support for all the amendments can be found throughout the specification and claims as originally filed. No new matter has been added a result of these amendment and entry thereof is respectfully requested.

Formalities

The Examiner has objected to the disclosure because of the presence of blank lines on pages 21, 22 and 27. By amendment herein the blank spaces have been removed.

In addition, in view of the foregoing amendments and attached Sequence Listing, the application fully complies with the requirements of 37 C.F.R. §§ 1.821-1.825.

35 U.S.C. § 112, First Paragraph

All pending claims stand rejected as allegedly not enabled by the specification as filed. The Examiner notes that the specification enables methods of using zinc finger proteins with a single regulatory domain and methods of delivering zinc finger proteins to cell in the form of expression vectors. (Office Action, page 4). However, it is alleged that the specification does not enable the use of two regulatory domains or delivery of zinc finger proteins by introduction of the protein itself. (Office Action, page 4).

Applicants traverse the rejections and address each in turn.

For the reasons detailed above and reiterated below, Applicants traverse this rejection. By law, a patent application is presumptively enabled when filed. *In re Marzocchi*, 169 USPQ 367, 369 (CCPA 1971). In other words, without reason to doubt the truth of the statements made in the patent application, the application must be considered enabling. See, *In re Wright*, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993) and *In re Marzocchi, supra*. The burden is on the Office to establish why the claimed invention is not enabled by the specification. Thus, the enablement requirement is satisfied if the

applicant's specification teaches one of skill in the art how to make and use the claimed invention without undue experimentation. *In re Wands,* 8 USPQ2d 1400 (Fed. Cir. 1988). When determining whether the amount of testing required is "undue", the courts have determined that "time and difficulty of experiments are not determinative if they are merely routine." (see, *e.g., In re Wands,* 8 USPQ2d at 1404, citing *In re Angstadt,* 190 USPQ 214 (CCPA 1976). In sum, Applicants reiterate that the test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation. *Ex parte Forman,* 230 USPQ 546 (P.T.O. Bd. Pat. App. & Int., 1986).

Regulatory Domains

The Examiner asserts that Applicants have provided guidance only with regard to a single regulatory domain having the desired regulatory effect. (Office Action, page 5). Therefore, it is maintained that it would require undue experimentation to use more than one regulatory domain.

Because Applicants' specification provides ample guidance regarding the use of more than one regulatory domain, Applicants traverse. Applicants remind the Examiner that working examples are not required by statute, rules or case law. (See, e.g., MPEP 2164.02). Rather, all that is required is that the specification teach a skilled artisan how to make and use the claimed invention. The specification at issue clearly satisfies this requirement. In this regard, Applicants direct the Examiner's attention to page 29 of the specification where, Applicants plainly teach that:

> The ZFP can be covalently or non-covalently associated with one or more regulatory domains, alternatively two or more regulatory domains, with the two or more domains being two copies of the same domain or two different domains. The regulatory domains can be covalently linked to the ZFP, e.g., via an amino acid linker as part of a fusion protein. The ZFPs can also be associated with the regulatory domain via a non-covalent dimerization domain, e.g., a leucine zipper, a STAT protein N terminal

domain, or an FK506 binding protein." (See, page 29, lines 2 to 7)

Furthermore, methods for construction of fusion proteins (or expression cassettes encoding such fusion proteins) are extensively taught by Applicants (see, for example, pages 33 to 36 and the Examples) and were well known to those in the field at the time of filing. Indeed, it would have been routine for someone of skill in the art following the teachings of the specification to make and use a fusion protein containing an engineered ZFP and two or more regulatory domains. Methods of making and using fusion polypeptides that include more then two functional components are exemplified by Applicants, for example on page 66 where generation and use of fusions comprising engineered ZFPs, a regulatory domain, a nuclear localization signal, and a FLAG epitope are described. Thus, the specification makes clear that, at the time of filing, it would have been routine to generate and use ZFPs linked to multiple functional domains. Because the evidence establishes that it would not require undue experimentation for one of skill in the art to make fusion proteins that include an engineered ZFP and two or more regulatory domains, Applicants respectfully request that the rejection be withdrawn.

Protein Delivery

Similarly, the specification fully enables one of skill in the art to practice the claimed methods using zinc finger proteins. "Administration" is clearly defined on page 15, lines 18 to 22 to include "any means by which a protein or nucleic acid can be transported across a cell membrane... ." Further, methods of delivering protein to cells are described in detail, for example, on page 43, line 31 to page 47, line 7, including such well known protein delivery methods such as (1) the use of membrane translocation polypeptides; (2) linkage to targeting peptides such as HIV tat, p16, a homeodomain of Antennapedia, the h region of a K-FGF signal peptide or the VP22 translocation domain of HSV; (3) use of toxins; or (4) use of liposome delivery techniques. As noted above,

actual working examples of these methods are not required in view of the fact that Applicants' specification in view of the state of art provide one of skill in the art with sufficient guidance as to delivery of protein forms of zinc finger proteins useful in the practice of the claimed methods.

In sum, when the factors in *Wands* are weighed, it would not require undue experimentation to practice the claimed invention, given the guidance found in the specification and state of the art. The claimed invention is, therefore, enabled.

35 U.S.C. 112, Second Paragraph

Claims 119-125, 135, 142-148, 158, 164-170 and 178 are rejected as allegedly indefinite. In particular, claims 121-125, 144-148 and 166-170 are alleged to have insufficient antecedent basis for the term "first zinc finger protein." (Office Action, page 6). Claims 119-121, 124, 125, 135, 142-144, 147, 148, 158, 164-166, 169, 170 and 178 are alleged to be indefinite because it is not clear that the second zinc finger protein is an engineered zinc finger protein. (Office Action, page 6). Applicants address each rejection in turn.

Claims 121-125, 144-148 and 166-170

Applicants thank the Examiner for bringing the typographical errors to Applicants' attention. By amendment herein, independent claims 118, 141 and 163 have been amended to indicate that the first zinc finger protein is engineered. Therefore, the rejection has been obviated.

Claims 119-121, 124, 125, 135, 142-144, 147, 148, 158, 164-166, 169, 170, 178

With regard to these particular claims, Applicants note that there is no requirement that the second zinc finger protein be engineered. In other words, while the

first zinc finger protein is engineered, the second zinc finger protein of the dependent claims can be engineered or non-engineered. Accordingly, Applicants submit that the claims are sufficiently definite and clear as filed.

In sum, the claims are now sufficiently definite and the rejections under 35 U.S.C. 112, second paragraph should be withdrawn.

35 U.S.C. § 103

Claims 118, 122, 126-130, 133, 134, 137, 138, 141, 145, 149-153, 156, 157, 159, 160, 163, 167, 171-174, 177, 180 and 181 stand rejected as allegedly unpatentable over Liu et al. (1996) PNAS (hereinafter "Liu '96") in view of WO 96/06166 (hereinafter "Choo") and in view of Liu et al. (1997) (hereinafter "Liu '97"). Claims 139, 161 and 182 stand rejected as allegedly obvious over Liu '96 in view of Choo and Liu '97 and in further view of Berg. Claims 131, 132, 154, 155 and 176 stand rejected as allegedly obvious over Liu '96 in view of Choo and Liu '97 and in further view of Berg and Mukhopadhyay et al. Claims 140, 162, and 183 stand rejected as allegedly obvious over Liu '96 in view of Choo and Liu '97 and in further view of Berg and Jones.

In support of these rejections, the Office Action states that Liu'96 discloses that the naturally-occurring zinc finger protein EGR-1 increases expression of TGF-β1 and causes reduced cell proliferation; that WO 96/06166 discloses methods to design a zinc finger protein that binds any desired target site and use of designed zinc finger proteins to alter expression of a desired target gene; and that Liu *et al.* '97 discloses engineered zinc finger proteins of high specificity. It is alleged that it would have been obvious to one of skill in the art to modify the naturally occurring zinc finger protein of Liu '96 using the methods of Choo and Liu '97. (Office Action, page 5).

In order to establish a *prima facie* case of obviousness the Office must show three things: (1) motivation within the references to arrive at the claimed invention; (2) a

reasonable expectation of success and (3) that the combination of references teaches each and every limitation of the claims. Each requirement is discussed in detail below. Applicants traverse the rejection because, regardless of what combination, the cited references do not teach or suggest the claimed invention.

1) There is no Motivation Within the References to Make the Suggested Changes

In order to establish a case of obviousness, the Office must show that there is a suggestion or motivation within the references that would lead a skilled artisan to modify (or combine) the references to arrive at the claimed invention. The Federal Circuit has repeatedly held that using "hindsight reconstruction" to provide the necessary motivation is improper. (see, e.g., *In re Fine,* 5 USPQ2d 1596, 1598 (Fed. Cir. 1988); *In re Napier* 34 USPQ2d 1782, 1784 (Fed. Cir. 1995) stating that "obviousness cannot be established by combining the teachings of the prior art to produce the claimed invention absent some teaching, suggestion or incentive supporting the combination."). In addition, the art must suggest the desirability of the modification. (*See, e.g., In re Gordon*, 221 USPQ 1125 (Fed. Cir. 1984), emphasis added, holding that "the mere fact that the prior art could be so modified would not have the modification obvious unless the prior art suggested the desirability of the modification"). It is insufficient for the Office to assert that the teachings of the references could be modified to arrive at the claimed invention when the proper standard is demonstrating that the cited references suggest the desirability of modifying their disclosures to arrive at the claimed invention. For the reasons detailed below, the cited references, alone or in combination, do not suggest the desirability of such modifications.

The pending claims are directed to methods of modulating (*e.g.,* activating or inhibiting) expression of an endogenous cellular gene using at least one engineered zinc finger protein. Such methods are not reasonably suggested by any of the cited references or combination of references. Indeed, the primary reference, Liu '96 not only fails to

provide the motivation to arrive at the claimed invention it actually teaches away from the claimed invention. Liu '96 is clearly directed only to use of naturally occurring ZFPs. There is no teaching or suggestion in Liu to make or use engineered ZFPs having modified binding specificity. Further, Liu '96 states, at page 11835, second column, last sentence of first (partial) paragraph: "Nevertheless, it is emphasized that it remains a hypothesis that the mechanism of induction of TGF-β1 secretion and growth suppression by EGR-1 involves direct binding of EGR-1 to the TGF-β1 gene promoter." In other words, Liu '96 does not describe, demonstrate or suggest that binding of any protein, let alone an engineered transcription factor, to a target site in a gene can be used in methods of modulating expression of an endogenous cellular gene.

The secondary references, Choo, Liu '97, Berg, Mukhopadhyay and Jones do not provide the requisite motivation to arrive at the claimed subject matter. Simply put, there is no suggestion in any of these references to regulate expression of an endogenous gene. Applicants' specification is clear -- an endogenous gene is one that is present in its native environment. See, page 5, lines 14-22; page 10, lines 17-20 and page 14, lines 16-30. In direct contrast, Choo is directed entirely toward libraries of zinc finger proteins and to methods of making and using these libraries on a non-endogenous bcr-abl gene. (Choo, Abstract). There is no indication in Choo that endogenous genes could be regulated. Similarly, there is no suggestion within Liu '97 to modulate expression of an endogenous gene.

Berg deals with the naturally-occurring SP1 zinc finger protein; Mukhopadhyay shows that VEGF expression is regulated by SP1; while Jones is directed to humanized antibodies. Thus, none of Choo, Liu '97, Berg, Mukhopadhyay and Jones teach or suggest methods of modulating expression of an endogenous cellular gene. Accordingly, there is nothing in the any of the secondary references that would suggest to one of skill in the art that the claimed invention would be desirable and nothing that would lead one of skill in the art to the claimed invention.

2) No Reasonable Expectation of Success

The second requirement that must be met by the Office is to show that the proposed modification of the references had a reasonable expectation of success, determined from the vantage point of the skilled artisan at the time the invention was made. (*See, e.g., Amgen v. Chugai,* 18 USPQ2d 1016 (Fed. Cir. 1991)). In moving from the prior art to the claimed invention, the Office cannot base a determination of "expectation of success" on what the skilled artisan might "try" or find "obvious to try." *In re Dow*, 5 USPQ2d 1529 (Fed. Cir. 1988).

Liu '96 is directed to use of a naturally occurring transcription factors and, moreover, fails entirely to teach or suggest engineered zinc finger proteins having modified binding specificity. Thus, to find the requisite reasonable expectation of success, the Office appears to be relying on the teachings of Liu '97 and Choo, which are cited as disclosing methods of designing zinc fingers. (Office Action, page 5). However, Applicants note that both Choo and Liu '97 are silent as to methods of modulating expression of an endogenous gene. In short, Liu '96 doesn't suggest modifying binding specificity and Choo and Liu '97 don't suggest modulating expression of endogenous genes. Thus, the combined teachings of the references do not address the claimed subject matter and, accordingly, the requisite reasonable expectation of success is lacking.

3) The Claimed Limitations are Not Suggested by the Combination of References

The third requirement which must be met by the Office is to show that the combination of references teaches or suggests all the limitations of the claims. *See, e.g., In re Vaeck,* 20 USPQ2d 1438 (Fed. Cir. 1991). The claims must also be considered as a whole and "focusing on the obviousness of substitutions and differences instead of on the invention as a whole ... [is] a legally improper way to simplify the difficult determination of obviousness." *Hybritech v. Monoclonal Antibodies Inc.,* 231 USPQ 81 (Fed. Cir. 1986). As with the first and second requirements, this third requirement has not been

met.

As a whole, the claims are directed to methods of activating or inhibiting (e.g., modulating) expression of an endogenous cellular gene. The combination of cited references does not teach or suggest this claimed invention. In particular, Liu '96 does not disclose critical elements, as this reference is limited to disclosure of a naturally occurring transcription factor to enhance expression, and is silent as to engineered zinc finger proteins that modulate expression. Choo and Liu '97 are silent as to methods of modulating endogenous gene expression. It is improper to focus on individual components of the invention, and to mix and match these components from the prior art and to conclude that the invention would have been obvious.

In sum, there is no combination of references that can result in the claimed subject matter. For all the foregoing reasons, Applicants submit that the Office has not established a *prima facie* case of obviousness and withdrawal of this rejection is respectfully requested.

Obviousness-Type Double Patenting

Two provisional double-patenting rejections have been asserted. First, claims 118-135, 137-178 and 180-183 have been provisionally rejected as unpatentable over various claims of co-pending Application No. 09/229,037. (Office Action, page 13). Second, claims 89-107, 113, 114, 116-129, 131-136, 142, 143, 145-163, 169- 170, 172 and 173 have been provisionally rejected under various claims of co-pending Application No. 09/229,037 and are alleged to represent a species of the instant claimed genus of a delivery method of a zinc finger to a target cell, and thus anticipates the claimed invention. (Office Action, page 14).

With regard to both rejections, Applicants note the pending application is a divisional of 09/229,037. The original claims co-pending Application No. 09/229,037 were subject to restriction/election as between methods involving protein delivery and

methods involving delivery of nucleic acids encoding such proteins. Accordingly, all the claims in the co-pending parent application are directed to delivery of a nucleic acid molecule encoding a zinc finger protein -- a species the Patent Office has already deemed distinct from that at issue. Thus, the provisional rejections are improper and should be withdrawn.

III. CONCLUSION

In view of the foregoing amendments, Applicants submit that the claims are now in condition for allowance and request early notification to that effect.

The Commissioner is hereby authorized to charge any fees under 37 C.F.R. §§ 1.16 and 1.17 which may be required by this paper, or to credit any overpayment, to Deposit Account No. 18-1648.

Respectfully submitted,

Date: April 17, 2002 By: Dahna Pasternak

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Marked-Up Specification Paragraphs:

Paragraph beginning on page 3, line 12, has been amended as follows:

Zinc finger proteins ("ZFPs") are proteins that can bind to DNA in a sequence-specific manner. Zinc fingers were first identified in the transcription factor TFIIIA from the oocytes of the African clawed toad, *Xenopus laevis*. ZFPs are widespread in eukaryotic cells. An exemplary motif characterizing one class of these proteins (C_2H_2 class) is -Cys-$(X)_{2-4}$-Cys-$(X)_{12}$-His-$(X)_{3-5}$-His (SEQ ID NO:1) (where X is any amino acid). A single finger domain is about 30 amino acids in length and several structural studies have demonstrated that it contains an alpha helix containing the two invariant histidine residues co-ordinated through zinc with the two cysteines of a single beta turn. To date, over 10,000 zinc finger sequences have been identified in several thousand known or putative transcription factors. ZFPs are involved not only in DNA-recognition, but also in RNA binding and protein-protein binding. Current estimates are that this class of molecules will constitute about 2% of all human genes.

Paragraph beginning on page 13, line 15, has been amended as follows:

The term "zinc finger protein" or "ZFP" refers to a protein having DNA binding domains that are stabilized by zinc. The individual DNA binding domains are typically referred to as "fingers" A zinc finger protein has least one finger, typically two fingers, three fingers, four fingers, five fingers, or six fingers or more. Each finger binds from two to four base pairs of DNA, typically three or four base pairs of DNA. A zinc finger protein binds to a nucleic acid sequence called a target site or target segment. Each finger typically comprises an approximately 30 amino acid, zinc-coordinating, DNA-binding subdomain. An exemplary motif characterizing one class of these proteins (Cys_2His_2 class) is -Cys-$(X)_{2-4}$-Cys-$(X)_{12}$-His-$(X)_{3-5}$-His (SEQ ID NO:1) (where X is any amino acid). Studies have demonstrated that a single zinc finger of this class consists of an

alpha helix containing the two invariant histidine residues co-ordinated with zinc along with the two cysteine residues of a single beta turn (*see, e.g.*, Berg & Shi, *Science* 271:1081-1085 (1996)).

On page 21, the paragraph beginning on line 10 has been changed as follows:

--The ZFPs of the invention are engineered to recognize a selectable target site in the endogenous gene of choice. Typically, a backbone from any suitable C_2H_2 (SEQ ID NO:1) ZFP, such as SP-1, SP-1C, or ZIF268 is used as the scaffold for the engineered ZFP (*see, e.g.*, Jacobs *EMBO J.* 11:4507 (1992); Desjarlais & Berg, *PNAS* 90:2256-2260 (1993)). A number of methods can then be used to design and select a ZFP with high affinity for its target (e.g., preferably with a K_d of less than about 25 nM). As described above, a ZFP can be designed or selected to bind to any suitable target site in the target endogenous gene, with high affinity. ~~Co-pending patent application USSN _____, filed January 12, 1999 (TTC docket no. 019496-001800, herein incorporated by reference)~~ Co-owned WO 00/42219, incorporated by reference herein in its entirety, comprehensively describes methods for design, construction, and expression of ZFPs for selected target sites.--

On page 21, please replace the paragraph beginning on line 32 with the following:

--In a preferred embodiment, co-owned WO 00/42219 provides methods that select a target gene, and identify a target site within the gene containing one to six (or more) D-able sites (see definition below). Using these methods, a ZFP can then be synthesized that binds to the preselected site. These methods of target site selection are premised, in part, on the recognition that the presence of one or more D-able sites in a target segment confers the potential for higher binding affinity in a ZFP selected or designed to bind to that site relative to ZFPs that bind to target segments lacking D-able sites. Experimental evidence supporting this insight is provided in Examples 2-9 of

~~copending application USSN _____, filed January 12, 1999 (TTC docket no. 019496-001800)~~ co-owned WO 00/42219.--

On page 22, please replace the paragraph beginning on line 9 with the following:

A D-able site or subsite is a region of a target site that allows an appropriately designed single zinc finger to bind to four bases rather than three of the target site. Such a zinc finger binds to a triplet of bases on one strand of a double-stranded target segment (target strand) and a fourth base on the other strand (*see* Figure 1 of ~~copending application USSN _____, filed January 12, 1999 (TTC docket no. 019496-001800)~~ co-owned WO 00/42219). Binding of a single zinc finger to a four base target segment imposes constraints both on the sequence of the target strand and on the amino acid sequence of the zinc finger. The target site with the target strand should include the "D-able" site motif 5' NNGK 3' (SEQ ID NO:41), in which N and K are convention IUPAC-IUB ambiguity codes. A zinc finger for binding to such a site should include an arginine residue at position -1 and an aspartic acid, (or less preferably a glutamic acid) at position +2. The arginine residues at position -1 interacts with the G residue in the D-able site. The aspartic acid (or glutamic acid) residue at position +2 of the zinc finger interacts with the opposite strand base complementary tot he K base in the D-able site. It is the interaction between aspartic acid (symbol D) and the opposite strand base (fourth base) that confers the name D-able site. AS is apparent from the D-able site formula, there are two subtypes of D-able sites; 5' NNGG 3' (SEQ ID NO:42) and 5' NNGT 3' (SEQ ID NO:43). For the former site, the aspartic acid or glutamic acid at position +2 of a zinc finger interacts with a C in the opposite strand to the D-able site. In the latter site, the aspartic acid or glutamic acid at position +2 of a zinc finger interacts with an A in the opposite strand of the D-able site. In general, NNGG (SEQ ID NO:42) is preferred over NNGT (SEQ ID NO:43).--

Paragraph beginning on page 23, line 12, has been amended as follows:

In the formula 5'-NNx aNy bNzc-3', the triplets of NNx aNy and bNzc represent the triplets of bases on the target strand bound by the three fingers in a ZFP. If only one of x, y and z is a G, and this G is followed by a K, the target site includes a single D-able subsite. For example, if only x is G, and a is K, the site reads 5'-**NNG KNy** bNzc-3' with the D-able subsite highlighted. If both x and y but not z are G, and a and b are K, then the target site has two overlapping D-able subsites as follows: 5'-**NNG *KNG* KNz** c-3' (SEQ ID NO:2), with one such site being represented in bold and the other in italics. If all three of x, y and z are G and a, b, and c are K, then the target segment includes three D-able subsites, as follows 5'**NNG *KNG* KNG K**3' (SEQ ID NO:3), the D-able subsites being represented by bold, italics and underline.

On page 27, please replace the paragraph beginning on line 8 with the following:

-- The biochemical properties of the purified proteins, e.g., K_d, can be characterized by any suitable assay. In one embodiment, K_d is characterized via electrophoretic mobility shift assays ("EMSA") (Buratowski & Chodosh, *in Current Protocols in Molecular Biology* pp. 12.2.1.-12.2.7 (Ausubel ed., 1996); *see also* U.S. Patent No. 5,789,538; ~~USSN _____, filed January 12, 1999 (TTC docket no. 019496-001800), herein incorpoated by reference, and~~ ~~Example 1 of~~ co-owned WO 00/42219 and Example 1, *infra.* Affinity is measured by titrating purified protein against a low amount of labeled double-stranded oligonucleotide target. The target comprises the nature binding site sequence (9 or 18 bp) flanked by the 3 bp found in the natural sequence. External to the binding site plus flanking sequence is a constant sequence. The annealed oligonucleotide targets possess a 1 bp 5' overhang which allows for efficient labeling of the target with T4 phage polynucleotide kinase. For the assay the target is added at a concentration of 40 nM or lower (the actual concentration is kept at least 10-fold lower than the lowest protein dilution) and the reaction is allowed to equilibrate for at least 45

min. In addition the reaction mixture also contains 10 mM Tris (pH 7.5), 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 5 mM DTT, 10% glycerol, 0.02% BSA (poly (dIdC) or (dAdT) (Pharamacia) can also be added at 10-100 µg/µl).--

Paragraph beginning on page 32, line 29, has been amended as follows:

Linker domains between polypeptide domains, e.g., between two ZFPs or between a ZFP and a regulatory domain, can be included. Such linkers are typically polypeptide sequences, such as polyglycine sequences of between about 5 and 200 amino acids. Preferred linkers are typically flexible amino acid subsequences which are synthesized as part of a recombinant fusion protein. For example, in one embodiment, the linker DGGGS (SEQ ID NO:4) is used to link two ZFPs. In another embodiment, the flexible linker linking two ZFPs is an amino acid subsequence comprising the sequence TGEKP (SEQ ID NO:5) (*see, e.g.*, Liu *et al.*, *PNAS* 5525-5530 (1997)). In another embodiment, the linker LRQKDGERP (SEQ ID NO:6) is used to link two ZFPs. In another embodiment, the following linkers are used to link two ZFPs: GGRR (SEQ ID NO:7) (Pomerantz *et al.* 1995, *supra*), $(G_4S)_n$ (SEQ ID NO:8) (Kim *et al.*, *PNAS* 93, 1156-1160 (1996.); and GGRRGGGS (SEQ ID NO:9); LRQRDGERP (SEQ ID NO:10); LRQKDGGGSERP (SEQ ID NO:11); LRQKD$(G_3S)_2$ ERP (SEQ ID NO:12). Alternatively, flexible linkers can be rationally designed using computer program capable of modeling both DNA-binding sites and the peptides themselves (Desjarlais & Berg, *PNAS* 90:2256-2260 (1993), *PNAS* 91:11099-11103 (1994) or by phage display methods.

Paragraph beginning on page 57, line 8, has been amended as follows:

This first Example demonstrates the construction of ZFPs designed to recognize DNA sequences contained in the promoter of the human vascular endothelial growth factor (VEGF) gene. VEGF is an approximately 46 kDa glycoprotein that is an endothelial cell-specific mitogen induced by hypoxia. VEGF has been implicated in

angiogenesis associated with cancer, various retinopathies, and other serious diseases. The DNA target site chosen was a region surrounding the transcription initiation site of the gene. The two 9 base pair (bp) sites chosen are found within the sequence agcGGGGAGGATcGCGGAGGCTtgg (SEQ ID NO:13), where the upper-case letters represent actual 9-bp targets. The protein targeting the upstream 9-bp target was denoted VEGF1, and the protein targeting the downstream 9-bp target was denoted VEGF3a. The major start site of transcription for VEGF is at the T at the 3' end of the first 9-bp target, which is underlined in the sequence above.

Paragraph beginning on page 58, line 22, has been amended as follows:

VEGF1 (SEQ ID NO:14):

GGTACCCATACCTGGCAAGAAGAAGCAGCACATCTGCCACATCCAGGGCTGT
GGTAAAGTTTACGGCACAACCTCAAATCTGCGTCGTCACCTGCGCTGGCACA
CCGGCGAGAGGCCTTTCATGTGTACCTGGTCCTACTGTGGTAAACGCTTCACC
CGTTCGTCAAACCTGCAGCGTCACAAGCGTACCCACACCGGTGAGAAGAAAT
TTGCTTGCCCGGAGTGTCCGAAGCGCTTCATGCGTAGTGACCACCTGTCCCGT
CACATCAAGACCCACCAGAATAAGAAGGGTGGATCC

Paragraph beginning on page 59, line 1, has been amended as follows:

VEGF1 translation (SEQ ID NO:15):

VPIPGKKKQHICHIQGCGKVYGTTSNLRRHLRWHTGERPFMCTWSYCGKRFTRS
SNLQRHKRTHTGEKKFACPECPKRFMRSDHLSRHIKTHQNKKGGS

Paragraph beginning on page 59, line 4, has been amended as follows:

VEGF3a (SEQ ID NO:16):

GGTACCCATACCTGGCAAGAAGAAGCAGCACATCTGCCACATCCAGGGCTGT
GGTAAAGTTTACGGCCAGTCCTCCGACCTGCAGCGTCACCTGCGCTGGCACA

CCGGCGAGAGGCCTTTCATGTGTACCTGGTCCTACTGTGGTAAACGCTTCACC
CGTTCGTCAAACCTACAGAGGCACAAGCGTACACACACCGGTGAGAAGAAAT
TTGCTTGCCCGGAGTGTCCGAAGCGCTTCATGCGAAGTGACGAGCTGTCACG
ACATATCAAGACCCACCAGAACAAGAAGGGTGGATCC

Paragraph beginning on page 59, line 11, has been amended as follows:

VEGF3a translation (SEQ ID NO:17):

VPIPGKKKQHICHIQGCGKVYGQSSDLQRHLRWHTGERPFMCTWSYCGKRFTRS
SNLQRHKRTHTGEKKFACPECPKRFMRSDELSRHIKTHQNKKGGS

Paragraph beginning on page 60, line 29, has been amended as follows:

VEGF site 1, top: 5'-CATGCATAGCGGGGAGGATCGCCATCGAT (SEQ ID NO:18)
VEGF site 1, bottom: 5'-ATCGATGGCGATCCTCCCCGCTATGCATG (SEQ ID NO:19)
VEGF site 3, top: 5'-CATGCATATCGCGGAGGCTTGGCATCGAT (SEQ ID NO:20)
VEGF site 3, bottom: 5'-ATCGATGCCAAGCCTCCGCGATATGCATG (SEQ ID NO:21)

Paragraph beginning on page 62, line 12, has been amended as follows:

An important consideration in ZFP design is DNA target length. For random DNA, a sequence of n nucleotides would be expected to occur once every 0.5×4^n base-pairs. Thus, DNA-binding domains designed to recognize only 9 bp of DNA would find sites every 130,000 bp and could therefore bind to multiple locations in a complex genome (on the order of 20,000 sites in the human genome). 9-bp putative repressor-binding sequences have been chosen for VEGF in the 5' UTR where they might directly interfere with transcription. However, in case zinc finger domains that recognize 9-bp sites lack the necessary affinity or specificity when expressed inside cells, a larger

domain was constructed to recognize 18 base-pairs by joining separate three-finger domains with a linker sequence to form a six-finger protein. This should ensure that the repressor specifically targets the appropriate sequence, particularly under conditions where only small amounts of the repressor are being produced. The 9-bp target sites in VEGF were chosen to be adjacent to one another so that the zinc fingers could be linked to recognize an 18-bp sequence. The linker DGGGS (SEQ ID NO:4) was chosen because it permits binding of ZFPs to two 9-bp sites that are separated by a one nucleotide gap, as is the case for the VEGF1 and VEGF3a sites (*see also* Liu *et al., PNAS* 5525-5530 (1997)).

Paragraph beginning on page 62, line 28, has been amended as follows:

The 6-finger VEGF3a/1 protein encoding sequence was generated as follows. VEGF3a was PCR amplified using the primers SPE7 (5'-GAGCAGAATTCGGCAAGAAGAAGCAGCAC (SEQ ID NO:22)) and SPEamp12 (5'-GTGGTCTAGACAGCTCGTCACTTCGC (SEQ ID NO:23)) to generate EcoRI and XbaI restriction sites at the ends (restriction sites underlined). VEGF1 was PCR amplified using the primers SPEamp13 (5'-GGAGCCAAGGCTGTGGTAAAGTTTACGG (SEQ ID NO:24)) and SPEamp11 (5'-GGAGAAGCTTGGATCCTCATTATCCC (SEQ ID NO:25)) to generate StyI and HindIII restriction sites at the ends (restriction sites underlined). Using synthetic oligonucleotides, the following sequence was ligated between the XbaI and StyI sites, where XbaI and StyI are underlined: TCT AGA CAC ATC AAA ACC CAC CAG AAC AAG AAA GAC GGC GGT GGC AGC GGC AAA AAG AAA CAG CAC ATA TGT CAC ATC CAA GG (SEQ ID NO:26). This introduced the linker sequence DGGGS (SEQ ID NO:4) between the two SP-1 domains. The ligation product was reamplified with primers SPE7 and SPEamp11 and cloned into pUC19 using the EcoRI and HindIII sites. The linked ZFP sequences were then amplified with primers

(1) GB19

GCCATGCCGGTACCCATACCTGGCAAGAAGAAGCAGCAC (SEQ ID NO:27)

(2) GB10

CAGATCGGATCCACCCTTCTTATTCTGGTGGGT (SEQ ID NO:28) to introduce KpnI and BamHI sites for cloning into the modified pMAL-c2 expression vector as described above.

Paragraph beginning on page 63, line 15 has been amended as follows:

The nucleotide sequence of the designed, 6-finger ZFP VEGF3a/1 from KpnI to BamHI is:

GGTACCCATACCTGGCAAGAAGAAGCAGCACATCTGCCACATCCAGGGCTGT
GGTAAAGTTTACGGCCAGTCCTCCGACCTGCAGCGTCACCTGCGCTGGCACA
CCGGCGAGAGGCCTTTCATGTGTACCTGGTCCTACTGTGGTAAACGCTTCACA
CGTTCGTCAAACCTACAGAGGCACAAGCGTACACACACAGGTGAGAAGAAA
TTTGCTTGCCCGGAGTGTCCGAAGCGCTTCATGCGAAGTGACGAGCTGTCTAG
ACACATCAAAACCCACCAGAACAAGAAAGACGGCGGTGGCAGCGGCAAAAA
GAAACAGCACATATGTCACATCCAAGGCTGTGGTAAAGTTTACGGCACAACC
TCAAATCTGCGTCGTCACCTGCGCTGGCACACCGGCGAGAGGCCTTTCATGTG
TACCTGGTCCTACTGTGGTAAACGCTTCACCCGTTCGTCAAACCTGCAGCGTC
ACAAGCGTACCCACACCGGTGAGAAGAAATTTGCTTGCCCGGAGTGTCCGAA
GCGCTTCATGCGTAGTGACCACCTGTCCCGTCACATCAAGACCCACCAGAAT
AAGAAGGGTGGATCC (SEQ ID NO:29)

Paragraph beginning on page 63, line 29, has been amended as follows:

The VEGF3a/1 amino acid translation (using single letter code) is:

VPIPGKKKQHICHIQGCGKVYGQSSDLQRHLRWHTGERPFMCTWSYCGKRFTRS
SNLQRHKRTHTGEKKFACPECPKRFMRSDELSRHIKTHQNKKDGGGSGKKKQHI

CHIQGCGKVYGTTSNLRRHLRWHTGERPFMCTWSYCGKRFTRSSNLQRHKRTH TGEKKFACPECPKRFMRSDHLSRHIKTHQNKKGGS (SEQ ID NO:30)

Paragraph beginning on page 64, line 1, has been amended as follows:

The 18-bp binding protein VEGF3a/1 was expressed in *E. coli* as an MBP fusion, purified by affinity chromatography, and tested in EMSA experiments as described in Example 1. The target oligonucleotides were prepared as described and comprised the following complementary sequences:

(1) JVF9

AGCGAGCGGGGAGGATCGCGGAGGCTTGGGGCAGCCGGGTAG (SEQ ID NO:31), and

(2) JVF10

CGCTCTACCCGGCTGCCCCAAGCCTCCGCGATCCTCCCCGCT (SEQ ID NO:32).

Paragraph beginning on page 65, line 23, has been amended as follows:

The VP16 protein of HSV-1 has been studied extensively, and it has been shown that the C-terminal 78 amino acids can act as a trans-activation domain when fused to a DNA-binding domain (Hagmann *et al., J. Virology* 71:5952-5962 (1997)). VP16 has also been shown to function at a distance and in an orientation-independent manner. For these studies, amino acids 413 to 490 in the VP16 protein sequence were used. DNA encoding this domain was PCR amplified from plasmid pMSVP16ΔC+119 using primers with the following sequences:

(1) JVF24

CGCGGATCCGCCCCCCCGACCGATG (SEQ ID NO:33), and

(2) JVF25

CCGCAAGCTTACTTGTCATCGTCGTCCTTGTAGTCGCTGCCCCCACCGTACTC GTCAATTCC (SEQ ID NO:34).

Paragraph beginning on page 66, line 5, has been amended as follows:

Three expression vectors were constructed for these studies. The general design is summarized in Figure 5. The vectors are derived from pcDNA3.1(+) (Invitrogen), and place the ZFP constructs under the control of the cytomegalovirus (CMV) promoter. The vector carries ampicillin and neomycin markers for selection in bacteria and mammalian cell culture, respectively. A Kozak sequence for proper translation initiation (Kozak, *J. Biol. Chem.* 266:19867-19870 (1991)) was incorporated. To achieve nuclear localization of the products, the nuclear localization sequence (NLS) from the SV40 large T antigen (Pro-Lys-Lys-Lys-Arg-Lys-Val (SEQ ID NO:35)) (Kalderon *et al., Cell* 39:499-509 (1984)) was added. The insertion site for the ZFP-encoding sequence is followed by the functional domain sequence. The three versions of this vector differ in the functional domain; "pcDNA-NKF" carries the KRAB repression domain sequence, "pcDNA-NVF" carries the VP16 activation domain, and "NF-control" carries no functional domain. Following the functional domain is the FLAG epitope sequence (Kodak) to allow specific detection of the ZFPs.

Paragraph beginning on page 66, line 19, has been amended as follows:

The vectors were constructed as follows. Plasmid pcDNA-ΔHB was constructed by digesting plasmid pcDNA3.1(+) (Invitrogen) with HindIII and BamHI, filling in the sticky ends with Klenow, and religating. This eliminated the HindIII, KpnI, and BamHI sites in the polylinker. The vector pcDNA3.1(+) is described in the Invitrogen catalog. Plasmid pcDNA-NKF was generated by inserting a fragment into the EcoRI/XhoI sites of pcDNA-ΔHB that contained the following: 1) a segment from EcoRI to KpnI containing the Kozak sequence including the initiation codon and the SV40 NLS sequence, altogether comprising the DNA sequence
GAATTCGCTAGCGCCACCATGGCCCCCAAGAAGAAGAGGAAGGTGGGAATC
CATGGGGTAC (SEQ ID NO:36),

where the EcoRI and KpnI sites are underlined; and 2) a segment from KpnI to XhoI containing a BamHI site, the KRAB-A box from KOX1 (amino acid coordinates 11-53 in Thiesen, 1990, *supra*), the FLAG epitope (from Kodak/IBI catalog), and a HindIII site, altogether comprising the sequence

GGTACCCGGGGATCCCGGACACTGGTGACCTTCAAGGATGTATTTGTGGACT
TCACCAGGGAGGAGTGGAAGCTGCTGGACACTGCTCAGCAGATCGTGTACAG
AAATGTGATGCTGGAGAACTATAAGAACCTGGTTTCCTTGGGCAGCGACTAC
AAGGACGACGATGACAAGTAAGCTTCTCGAG (SEQ ID NO:37)

where the KpnI, BamHI and XhoI sites are underlined.

Paragraph beginning on page 67, line 14, has been amended as follows:

The effector plasmids used in Example V were constructed as follows. Plasmid pcDNA-NVF was constructed by PCR amplifying the VP16 transactivation domain, as described above, and inserting the product into the BamHI/HindIII sites of pcDNA-NKF, replacing the KRAB sequence. The sequence of the inserted fragment, from BamHI to HindIII, was:

GGATCCGCCCCCCCCGACCGATGTCAGCCTGGGGGACGAGCTCCACTTAGACG
GCGAGGACGTGGCGATGGCGCATGCCGACGCGCTAGACGATTTCGATCTGGA
CATGTTGGGGGACGGGGATTCCCCGGGGCCGGGATTTACCCCCCACGACTCC
GCCCCCTACGGCGCTCTGGATATGGCCGACTTCGAGTTTGAGCAGATGTTTAC
CGATGCCCTTGGAATTGACGAGTACGGTGGGGGCAGCGACTACAAGGACGAC
GATGACAAGTAAGCTT (SEQ ID NO:38).

Paragraph beginning on page 67, line 28, has been amended as follows:

The effector plasmids used in Example VI were constructed as follows. Plasmid NF-control was generated by inserting the sequence

GAATTCGCTAGCGCCACCATGGCCCCCAAGAAGAAGAGGAAGGTGGGAATC

CATGGGGTACCCGGGGATGGATCCGGCAGCGACTACAAGGACGACGATGAC
AAGTAAGCTTCTCGAG (SEQ ID NO:39)
into the EcoRI-XhoI sites of pcDNA-NKF, thereby replacing the NLS-KRAB-FLAG sequences with NLS-FLAG only.

Paragraph beginning on page 68, line 31, has been amended as follows:

The reporter plasmid system was based on the pGL3 firefly luciferase vectors (Promega). Four copies of the VEGF target sites were inserted upstream of the SV40 promoter, which is driving the firefly luciferase gene, in the plasmid pGL3-Control to create pVFR1-4x. This plasmid contains the SV40 enhancer and expresses firefly luciferase to high levels in many cell types. Insertions were made by ligating together tandem copies of the two complementary 42-bp oligonucleotides, JVF9 and JVF10, described in Example 2. Adaptor sequences were ligated on, and the assembly was inserted into the MluI/BglII sites of pGL3-Control. This resulted in the insertion of the following sequence between those sites:
ACGCGTaagcttGCTAGCGAGCGGGGAGGATCGCGGAGGCTTGGGGCAGCCGGG
TAGAGCGAGCGGGGAGGATCGCGGAGGCTTGGGGCAGCCGGGTAGAGCGAG
CGGGGAGGATCGCGGAGGCTTGGGGCAGCCGGGTAGAGCGAGCGGGGAGGA
TCGCGGAGGCTTGGGGCAGCCGGGTAGAGCGCTCAGaagcttAGATCT (SEQ ID NO:40).

Version Showing Changes Made to Claims

118. (Amended) A method of inhibiting expression of an endogenous cellular gene in a cell, the method comprising the step of:

contacting a first target site in the endogenous cellular gene with [an engineered] a first zinc finger protein, wherein said first zinc finger protein is engineered and wherein the Kd of the zinc finger protein is less than about 25 nM;

thereby inhibiting expression of the endogenous cellular gene.

141. (Amended) A method of activating expression of an endogenous cellular gene, the method comprising the step of:

contacting a first target site in the endogenous cellular gene with [an engineered] a first zinc finger protein, wherein said first zinc finger protein is engineered and wherein the Kd of the zinc finger protein is less than about 25 nM;

thereby activating expression of the endogenous cellular gene.

163. (Amended) A method of modulating expression of an endogenous cellular gene in a cell, the method comprising the step of:

contacting a first target site in the endogenous cellular gene with [an engineered] a first zinc finger protein, wherein said first zinc finger protein is engineered;

thereby modulating expression of the endogenous cellular gene.